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14. ABSTRACT
During the past grant period, we obtained further evidence that eukaryotic elongation factor-2 kinase (eEF-2K) plays an important role in the development and progression of cancer by regulating cellular metabolism. In the study of the molecular mechanisms and pathways by which eEF-2K promotes glycolysis, we demonstrated that c-Myc-PKM2 pathway mediated the effect of eEF-2K on glycolysis. We further showed that suppression of eEF-2K impeded the induction of glycolysis in tumor cells subjected to hypoxia, enhanced apoptosis, and sensitized breast cancer cells to hypoxic insult. The results obtained during the last grant period have identified eEF-2K as a critical regulator of cell proliferation by promoting cancer cell metabolism, and provide new evidence that the effect of eEF- 2K on glycolysis is mediated through PKM2 and c-Myc. In addition, depletion of eEF-2K prevented the hypoxia-induced glycolysis and enhanced the sensitivity of tumor cells to hypoxic stress. These results underscore the potential of eEF-2K as a target for prevention and treatment of breast cancer.

15. SUBJECT TERMS- Elongation factor-2 kinase; pyruvate kinase M2; energy metabolism; apoptosis; breast cancer

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INTRODUCTION

Cancer cells rely mainly on aerobic glycolysis to generate ATP instead of mitochondrial electron transport, resulting in an increased rate of glucose uptake and lactate production, even in the presence of sufficient oxygen supply (1). This phenomenon was first described by Dr. Otto Warburg, and known as the Warburg effect. Glycolytic cancer cells are believed to be resistant to anticancer treatment and to induction of apoptosis mediated through mitochondrial function. In addition, glycolytic cancers are also more invasive (2). These make glycolytic metabolism in cancers as an attractive target for therapy development. Our group previously reported that eukaryotic elongation factor-2 kinase (eEF-2K), a negative regulator of protein synthesis (3), plays an important role in cell survival (4), and is overexpressed in breast cancer cell lines and human breast cancer specimens (5). During the first grant period, we found that inhibition of eEF-2 kinase by siRNA significantly decreased ATP and lactate levels in three human breast cancer cell lines, supporting a role for eEF-2K in activating glycolysis in breast cancer cells. In the past grant period, we progressed to investigate the regulatory mechanisms of glycolysis by eEF-2K in breast cancer cells. We found that pyruvate kinase M2 isoform (PKM2) is involved in the regulation of glycolysis by eEF-2K in breast cancer cells. Pyruvate kinase, an enzyme for the last step of glycolysis, catalyzes the production of pyruvate and ATP from phophoenolpyruvate (PEP) and ADP (6). There are four PK isoforms in mammals: L, R, M1 and M2. PKM2 contributes to the metabolism shift from oxidative phosphorylation to aerobic glycolysis and tumorigenesis, and is found predominantly in tumor cells (7). We showed that silencing of eEF-2K expression caused down-regulations of the mRNA, protein and activity of PKM2. Furthermore, we demonstrated that the repression of PKM2 transcription in the eEF-2K knockdown cells could be rescued by ectopic expression of c-Myc, supporting a role of the c-Myc-PKM2 pathway in mediating the effect of eEF-2K on glycolysis. Thus, this study not only uncovered a new function of eEF-2K in cancer, but also identified a potentially important determinant that controls glycolysis in cancer cells.

BODY

TRAINING

Mentoring:

According to my training plan, I meet with my primary mentor weekly, and the co-mentor every two months to discuss project goals. In the past one year, I have also had a meeting with my mentoring committee to discuss the research plan and progress.

Management and Career Independence:

In the past grant period, I attended the following courses:

Penn State Hershey Post-doctoral Society Workshop*:*

How to improve your CV & recommendation letters **(**November 13, 2012)

Negotiation workshop (December 6, 2012)

Professional development workshop on grant writing (May 29, 2013)

Courses and Training Programs:

In the past grant period, I attended the following courses and program:

Biochemistry 510, Penn State College of Medicine

Nature Miami Symposium: The molecular basis of metabolisms and nutrition (Feb 10~Feb 13, 2013)

18th Annual Penn State Hershey Cancer Institute Symposium (Oct 25, 2012)

Cancer Institute Interdisciplinary Conference Series (monthly)

Department of Pharmacology Seminar Series (weekly)

The Experimental Therapeutics Program's Monthly Meeting

Penn State Cancer Institute Breast Cancer Focus Group Meeting and Penn State Cancer Institute & Division of Medical Oncology (weekly).

RESEARCH

Task 1 To determine the role of eEF-2K in tumor cell grow.

In the first grant period, I found that eEF-2K plays a critical regulatory role in cellular energy metabolism in breast cancer cells. Because tumor cells rely mainly on glycolysis to generate energy, we also wanted to know whether eEF-2K has a role in tumor cell growth and proliferation. To investigate the role of eEF-2K in tumor cell proliferation, we used H-Ras^{V12}-transformed mouse embryonic fibroblast (MEF) cells with wild-type (+/+) or homozygous disruption of eEF-2K (-/-) (Fig. 1A). As shown in Fig. 1B, H-Ras^{V12}-eEF-2K$^{-/-}$ MEF cell line proliferated significantly slower than H-Ras^{V12}-eEF-2K$^{+/+}$ MEFs. To extend these observations to human tumor cells, we knocked down eEF-2K in human breast cancer MCF-7 cells using a small hairpin RNA (shRNA) targeting this kinase. We found that MCF-7 cells with eEF-2K knockdown showed a decreased proliferation rate and formed less colonies, as compared to control cells without eEF-2K knockdown (Fig. 1C and D). These observations indicate that eEF-2K is required for active cellular proliferation and tumor formation by regulating cell metabolism.

Task 2 To determine how eEF-2K promotes cell metabolism in breast cancer cells.

In order to explore the molecular mechanisms by which eEF-2K promotes glycolysis, we examined the effects of eEF-2K on several key players in the glycolytic pathway. Among these proteins, PKM2 was found to participate in the eEF-2K-regulated glycolysis. We showed that inhibition of eEF-2K led to a noticeable decline in both of the activity and protein expression of PKM2 (Fig. 2A and B). We also measured the level of PKM2 mRNA, and found that knockdown of eEF-2K led to a reduction in PKM2 mRNA expression, indicating that eEF-2K impacts PKM2 at the transcription level (Fig. 2C). In addition, the amount of pyruvate, the product of a reaction catalyzed by PKM2, was also decreased in eEF-2K knockdown cells (Fig. 2D). These results suggest that the effect of eEF-2K on glycolysis is mediated through PKM2. Furthermore, we found that c-Myc, a transcription factor, played a role in the transcriptional regulation of PKM2 in the cancer cells with silencing of eEF-2K expression, as

evidenced by a reduction of PKM2 mRNA in c-Myc knockdown cells (Fig. 3A). Moreover, overexpression of c-Myc led to an increase in the PKM2 mRNA expression (Fig. 3B). The repression of PKM2 transcription in the eEF-2K knockdown cells could be rescued by ectopic expression of c-Myc (Fig. 3C). These results raised the possibility that c-Myc-PKM2 pathway mediates the effect of eEF-2K on glycolysis. Studies are in progress to further verify and define the molecular mechanism involved.

Task 3 To determine the impact of targeting eEF-2K-mediated cellular energetics on breast cancer survival subjected to hypoxia.

Under hypoxia, cells switch from aerobic to anaerobic metabolism to meet the energy requirements for survival and proliferation (8). Indeed, hypoxic cancer cells activate glucose uptake and glycolysis to produce pyruvate, which is then converted into lactate instead of being oxidized via the tricarboxylic acid cycle and oxidative phosphorylation (9). To determine the impact of eEF-2K-mediated energy metabolism on breast cancer survival under hypoxic condition, we next tested whether eEF-2K played a regulatory role in PKM2 in breast cancer cells subjected to hypoxia. In these experiments, we compared the mRNA expression and activity of PKM2, and pyruvate production, in the cells with or without silencing of eEF-2K expression under normoxia or hypoxia. As shown in Fig. 4, knockdown of eEF-2K blocked the activation of PKM2 induced by hypoxia in breast cancer cells. In addition, eEF-2K knockdown inhibited the induction of glycolysis by hypoxia in breast cancer cells, as indicated by a decreased in lactate production and ATP levels (Fig. 5A and B). Silencing of eEF-2K expression also increased sensitivity of breast cancer cells to hypoxia, and augmented apoptosis in the hypoxic cancer cells (Fig. 5C and D). These data indicate that inhibition of eEF-2K can sensitize breast cancer cells to hypoxia by down-regulating cellular metabolism.

KEY RESEARCH ACCOMPLISHMENTS

• We found that down-regulation of eEF-2K caused a reduction of proliferation in human breast cancer cells and transformed MEFs.

• We showed that PKM2 was a key player in the eEF-2K-mediated regulation of glycolysis in tumor cells.

• We obtained evidence showing that c-Myc played a critical role in the eEF-2K-mediated regulation of PKM2 through modulating the transcription of PKM2.

• We demonstrated that down-regulation of eEF-2K inhibited the induction of glycolysis in hypoxic tumor cells.

• We observed that suppression of eEF-2K enhanced apoptotic activity and sensitized breast cancer cells to hypoxia.

REPORTABLE OUTCOMES

Abstract

Cheng Y, Ren XC, Zhang L, Yang JM: Identification of eukaryotic elongation factor-2 kinase as a critical regulator of Warburg effect. **Nature Miami Symposium: The Molecular Basis of Metabolism and Nutrition**. 2013.

Published Papers

Cheng Y, Ren XC, Zhang Y, Shan Y, Huber-Keener KJ, Zhang L, Kimball SR, Harvey H, Jefferson LS, Yang JM. Integrated Regulation of autophagy and apoptosis by EEF2K controls cellular fate and modulates the efficacy of curcumin and velcade against tumor cells. **Autophagy**. 2013, 9: 208-219.

Cheng Y, Ren XC, Gowda P, Shan Y, Zhang L, Yuan YS, Patel R, Wu H, Huber-Keener KJ, Yang JW, Liu D, Spratt TE, Yang JM. Interaction of sirt3 with OGG1 contributes to repair of mitochondrial DNA and protects from apoptotic cell death under oxidative stress. **Cell Death & Disease**. 2013, 4: e731.

Cheng Y, Ren XC, Hait WN, Yang JM. Therapeutic targeting of autophagy in disease: biology and pharmacology. **Pharmacol Rev.** 2013, 65: 1162-1197.

CONCLUSIONS

The results of my study during the last grant period provide new evidence that eEF-2 kinase is a positive regulator of cellular metabolism in breast cancer cells, and that eEF-2K plays a critical role in both of development and maintenance of malignant phenotype by regulating cancer cell metabolism. c-Myc-PKM2 pathway mediates the effect of eEF-2K on glycolysis. Inhibition of eEF-2K also prevented the hypoxia-induced glycolysis and enhanced the sensitivity of tumor cells to hypoxic stress.

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APPENDIX

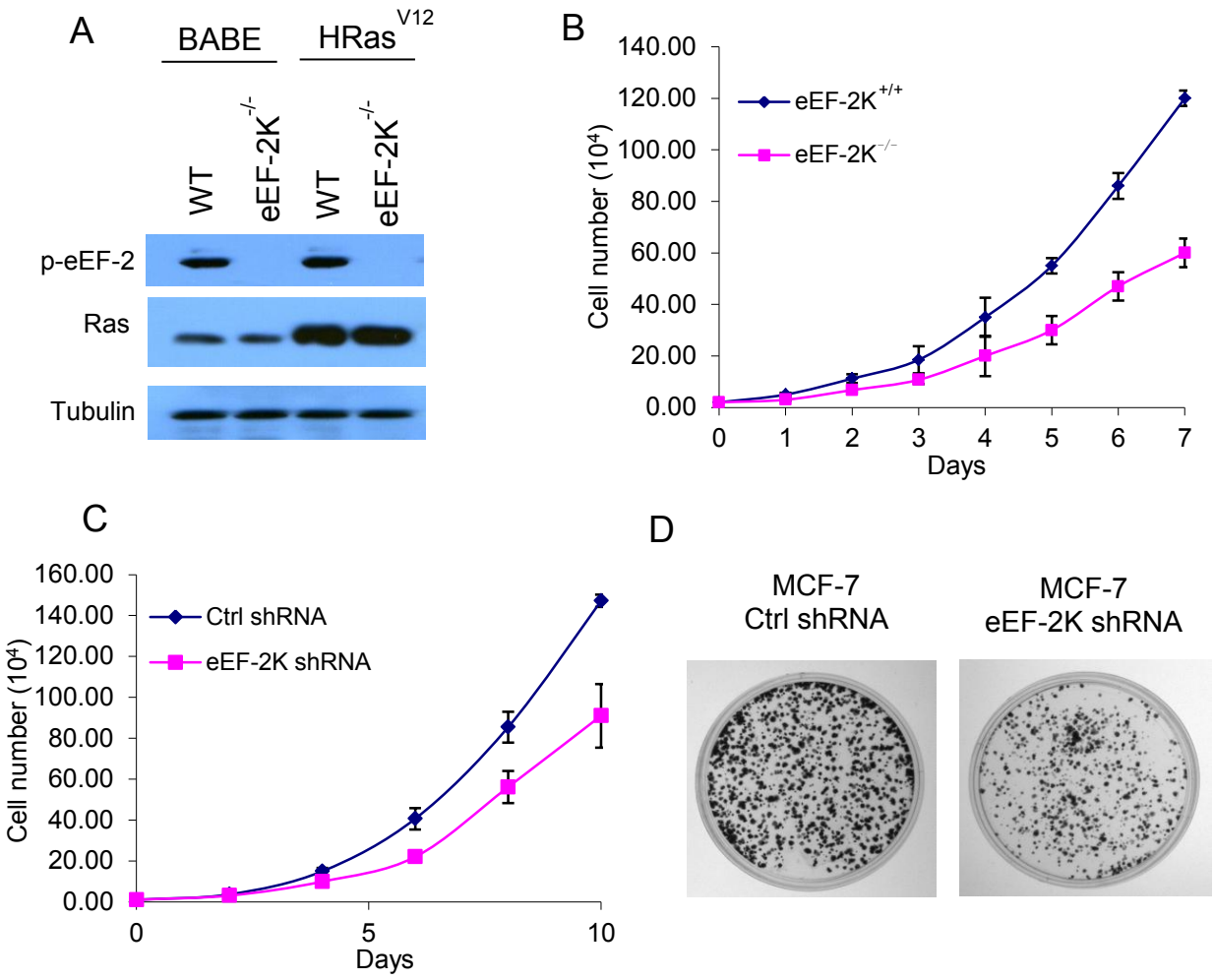


Figure 1. Effect of eEF-2K on cell proliferation in Ras-transformed epithelial cells and human breast cancer cells. **(A)** The expressions of Ras and p-eEF-2 were determined by Western blot in eEF-2K$^{+/+}$ and eEF-2K$^{-/-}$ MEFs expressing empty vector or H-Ras^{V12}. Tubulin was used as a loading control. (B) Proliferation of H-Ras^{V12}-eEF-2K$^{-/-}$ MEFs and H-Ras^{V12}-eEF-2K$^{+/+}$ MEFs. (C) Proliferation of MCF-7 cells stably expressing control or eEF-2K shRNA. (D) Colony formation of MCF-7 cells stably expressing control or eEF-2K shRNA.

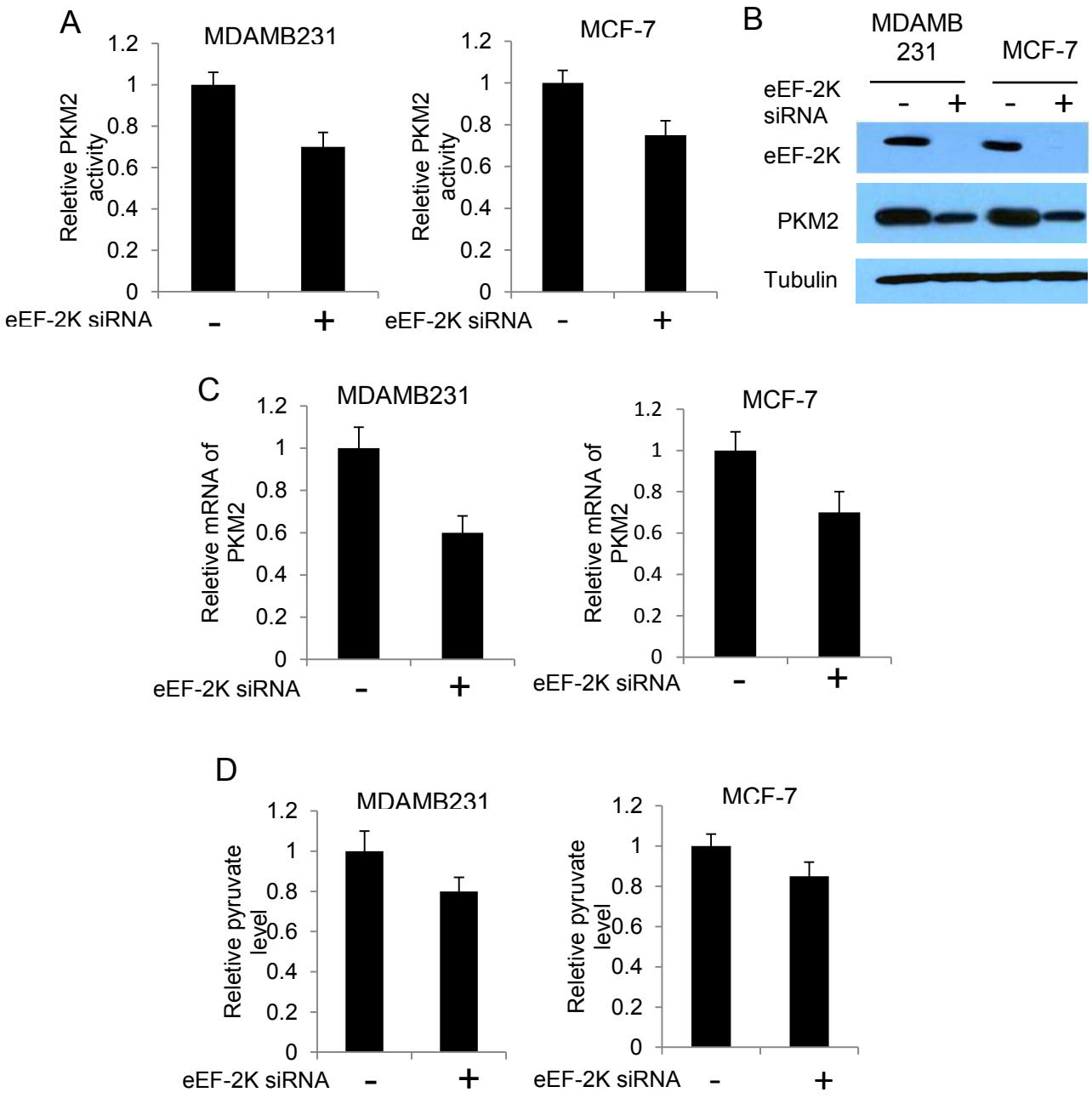


Figure 2. Effects of eEF-2K on activity, protein and mRNA expression of PKM2 in human breast cancer cells. MDAMB231 and MCF-7 cells were transfected with a non-targeting RNA or a siRNA targeting eEF-2 kinase. (A) PKM2 activity, (B) The protein expressions of eEF-2K and PKM2, (C) PKM2 mRNA level, and (D) Pyruvate level, were measured. Each bar represents the mean ± SE of three experiments.

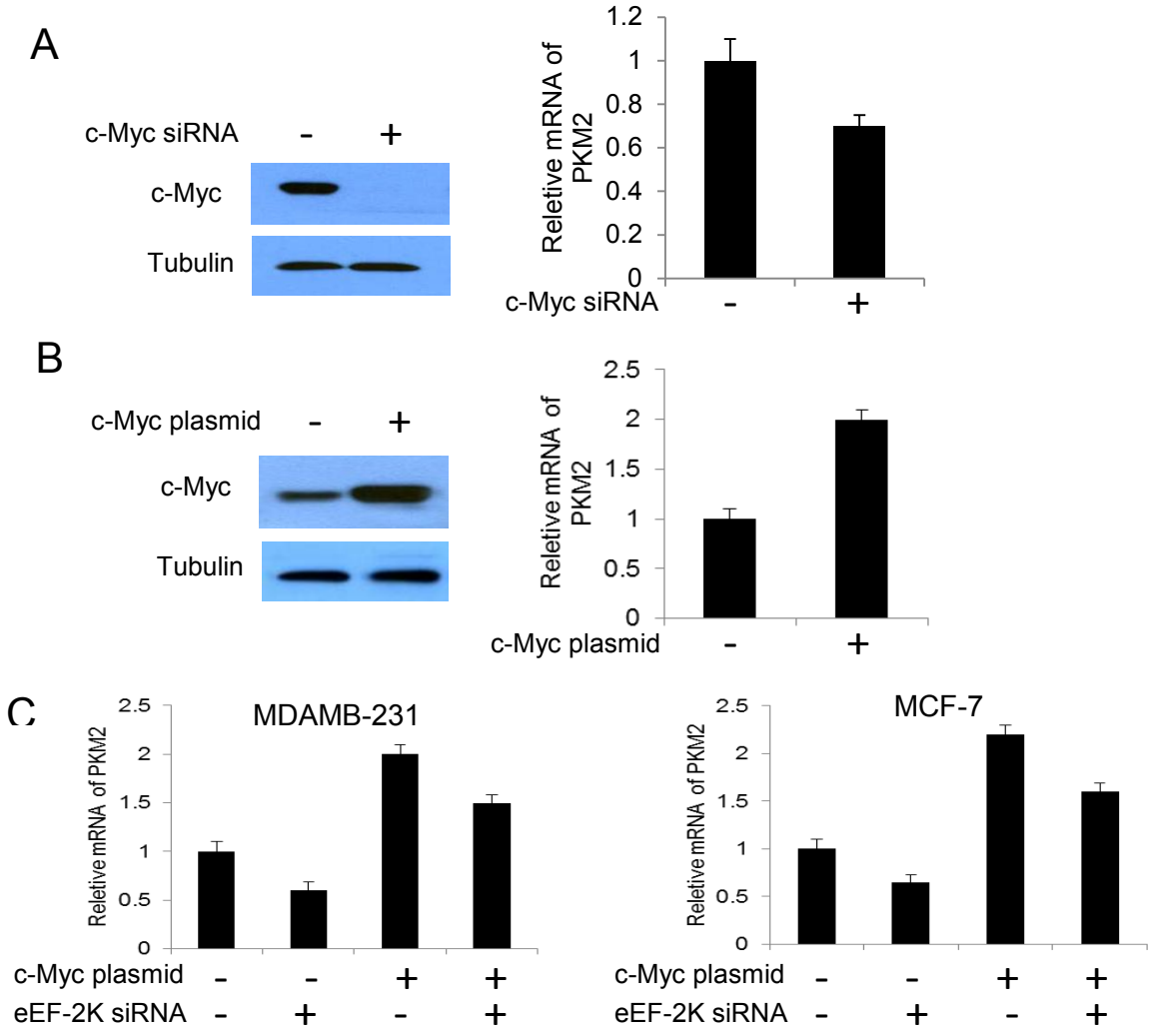


Figure 3. c-Myc regulates the transcription of PKM2, and c-Myc overexpression rescues the down-regulation of PKM2 mRNA by eEF-2K knockdown. (A) MDAMB-231 cells were transfected with a non-targeting or c-Myc siRNA. c-Myc protein expression was examined by Western blot. Tubulin was used as a loading control. The expression of PKM2 mRNA was measured by qRT-PCR. **(B)** MDAMB-231 cells were transfected with a control vector or c-Myc plasmid. c-Myc protein expression was examined by Western blot. Tubulin was used as a loading control. The expression of PKM2 mRNA was measured by qRT-PCR. **(C)** MDAMB-231 and MCF-7 cells were transfected with a non-targeting or eEF-2K siRNA, followed by c-Myc plasmid transfection. The expression of PKM2 mRNA was measured by qRT-PCR.

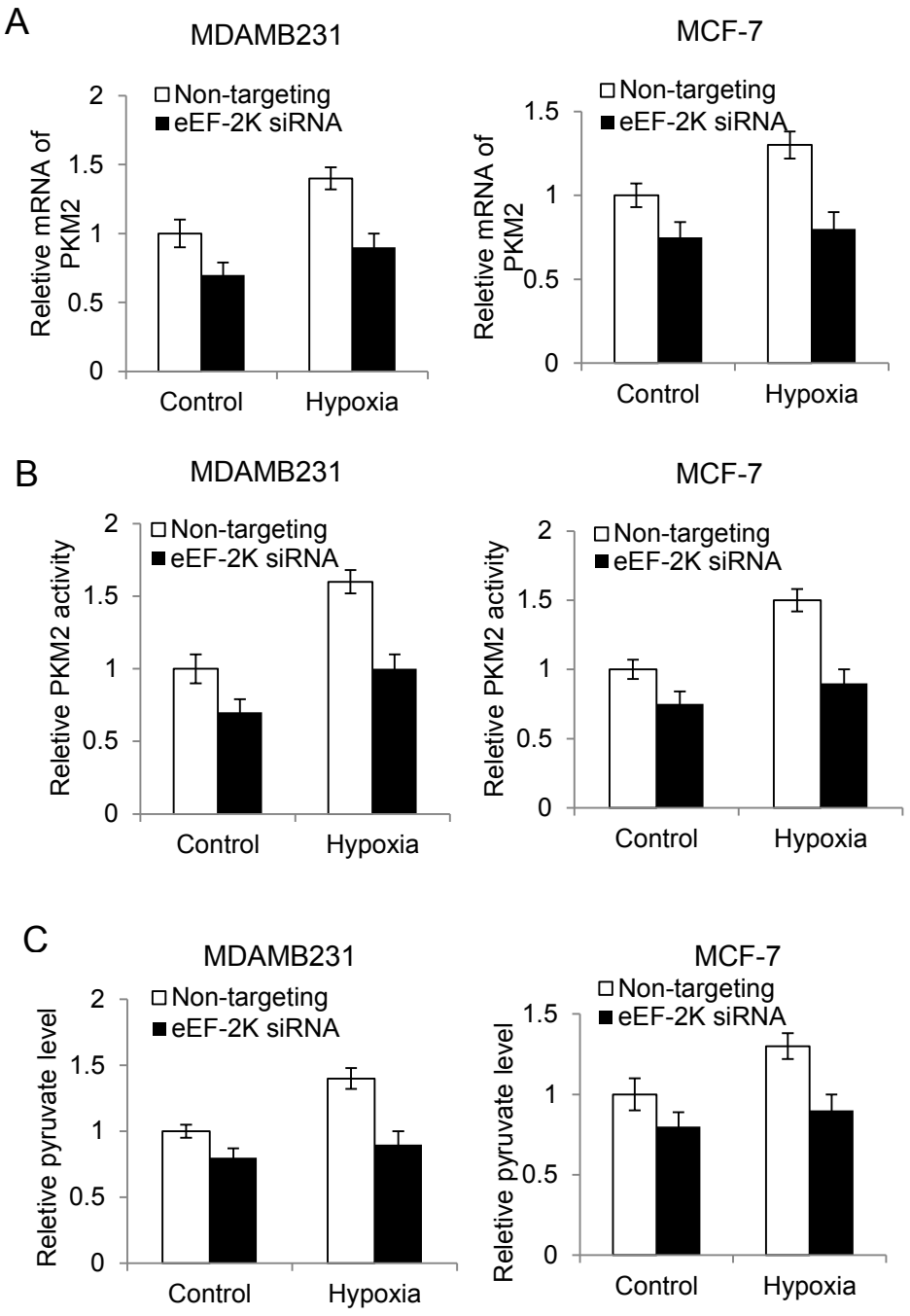
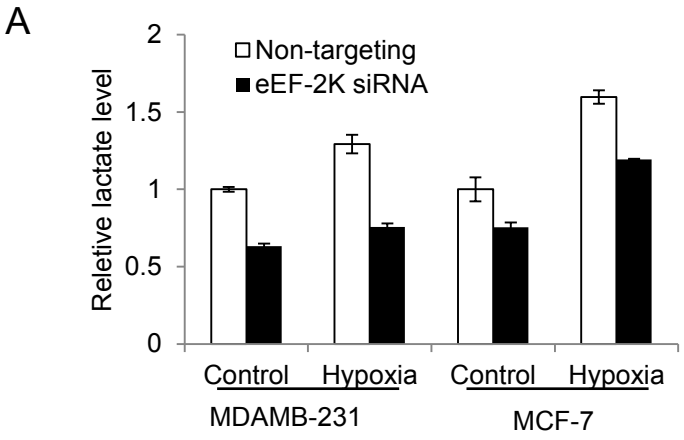
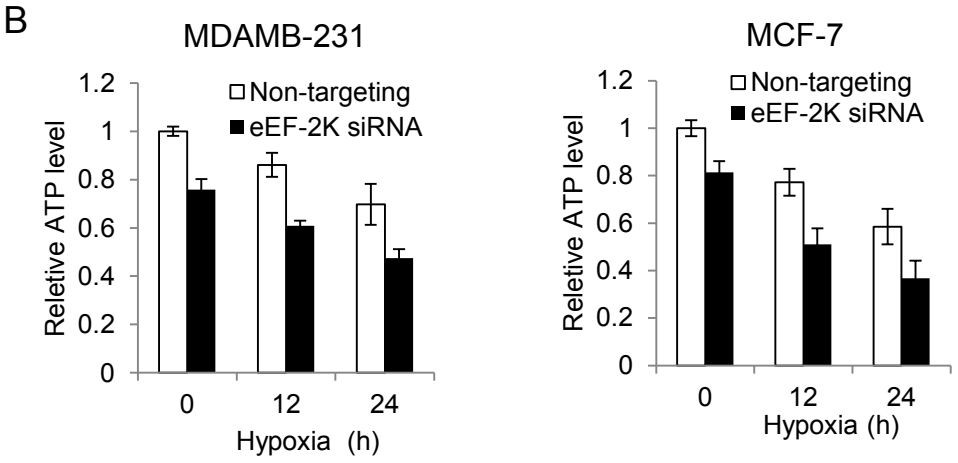
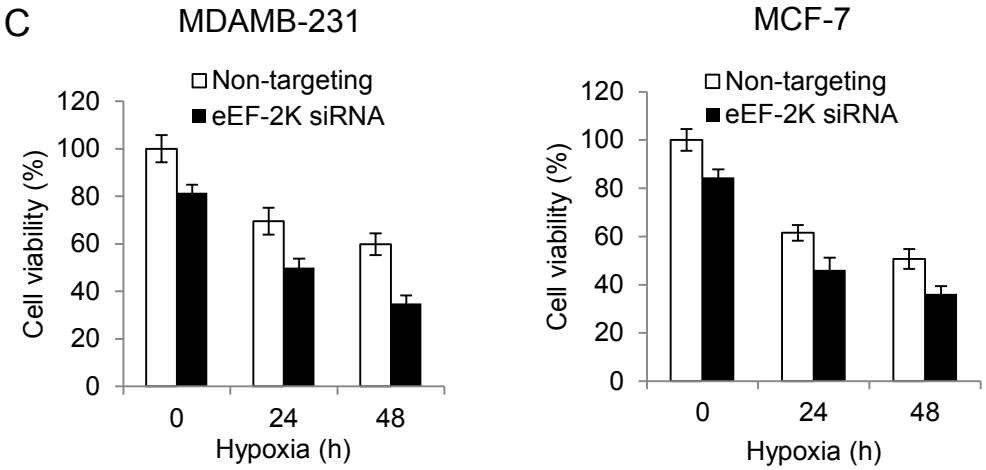


Figure 4. Effects of eEF-2K on the mRNA expression and activity of PKM2 in human breast cancer cells subjected to hypoxia. MDA-MB-231 and MCF-7 cells were transfected with a siRNA targeting eEF-2 kinase or a non-targeting RNA, and then treated with hypoxia. **(A)** PKM2 mRNA, **(B)** PKM2 activity, and **(C)** Pyruvate level, were measured. Each bar represents the mean ± SE of three experiments.


A
Non-targeting
eEF-2K siRNA
Reletive lactate level
2
1.5
1
0.5
0
Control
Hypoxia
Control
Hypoxia
MDAMB-231
MCF-7


B
MDAMB-231
MCF-7
Non-targeting
eEF-2K siRNA
Reletive ATP level
1.2
1
0.8
0.6
0.4
0.2
0
0
12
24
Hypoxia (h)


C
MDAMB-231
MCF-7
Non-targeting
eEF-2K siRNA
Cell viability (%)
120
100
80
60
40
20
0
0
24
48
Hypoxia (h)

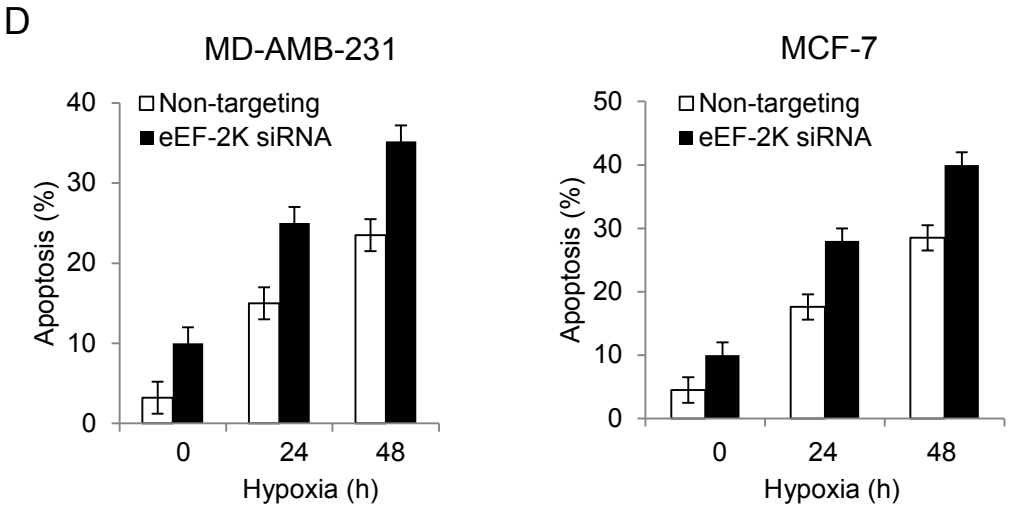


Figure 5. Silencing of eEF2K expression results in decreased glycolysis, and enhances sensitivity of breast cancer cells in response to hypoxia. MDAMB231 and MCF7 cells were transfected with non-targeting RNA or eEF2K siRNA, followed by treatment with hypoxia. **(A)** Lactate level, **(B)** ATP level, **(C)** Cell viability, and **(D)** Apoptosis, were measured. Each bar represents the mean ± SE of three experiments.